

Effectiveness of Bt Maize against Corn Rootworm (Coleoptera: Chrysomelidae) and Species Composition in South Dakota Ten Years Following the Introduction of Transgenic Maize¹

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ABSTRACT Corn rootworms, *Diabrotica* spp. (Coleoptera: Chrysomelidae), are the most economically important pest of maize in the United States. The larvae consume maize roots, contributing to poor nutrient uptake, lodging of the plants, and severe yield loss. Transgenic maize expressing toxins from the bacterium *Bacillus thuringiensis* (Bt) have been employed to combat the pest. However, corn rootworm resistance to Bt maize has recently been reported and spurred research on the effectiveness of Bt toxins against corn rootworm across the Corn Belt. Thus, our objectives were twofold. First, we quantified the efficacy of Bt maize hybrids against corn rootworm in South Dakota. Second, we surveyed populations of *Diabrotica* species in areas where Bt maize had been grown since its commercialization in 2003. Maize hybrids expressing Cry3Bb1, Cry34/35Ab1, and mCry3A were evaluated at two locations with previous corn rootworm infestations or in fields that were in continuous maize for three or more consecutive years. We reported damage to maize expressing Cry3Bb1 and mCry3A toxins. We also observed significantly higher numbers of western corn rootworm than northern corn rootworm in our *Diabrotica* species surveys, suggesting that western corn rootworm are the dominant species in the region. This research has implications for maize production and sustainable corn rootworm management in South Dakota and the Northern Plains, and will advance our knowledge of the incidence of resistance to Bt toxins and long-term impact of Bt hybrids on species diversity of *Diabrotica*.

KEY WORDS *Diabrotica virgifera virgifera*, *Diabrotica barberi*, Cry3Bb1, mCry3A, Cry34/35Ab1

The United States is home to several native species of *Diabrotica* (Coleoptera: Chrysomelidae), and two of them are key pests of maize (Chiang 1973). The northern corn rootworm, *Diabrotica barberi* Smith & Lawrence, was first collected in Colorado in 1820 (Say 1824) and was first reported as a pest after being found in maize fields in Stark County, Illinois, in July of 1880 (Thomas 1881). The western corn rootworm, *Diabrotica virgifera virgifera* Leconte, was first collected in Kansas in 1867 (LeConte 1868) and described as a pest of maize

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from reports in Colorado recorded in 1909 (Gillette 1912). Both species of corn rootworm have similar life cycles. Adults feed on maize pollen, silks, leaf tissue, and kernels (Gray et al. 2009), while larvae feed on maize roots, complete three instars, and pupate for about seven days at the base of the maize plant (Spencer 2010).

While maize is the preferred host of corn rootworm, a select few other grasses can support larval development and adult emergence. For example, in greenhouse studies *D. barberi* larvae were recovered when fed slender wheatgrass, *Elymus trachycaulus* (Link) Gould ex Shinnars (Poaceae); Canada wildrye, *Elymus canadensis* L.; and western wheatgrass, *Pascopyrum smithii* (Rydb.) Á.Löve (Poaceae) (Oyediran et al. 2008). Moreover, larvae of *D. v. virgifera* were able to develop to adults when fed on western wheatgrass; pubescent wheatgrass, *Elytrigia intermedia* (Host) Nevski (Poaceae); and prairie cordgrass, *Spartina pectinata* Bosc ex Link (Poaceae) (Oyediran et al. 2004). However, significant corn rootworm populations can only be sustained in areas where maize is grown continuously (Spencer 2010).

The larval stage of the corn rootworm is the most damaging to maize. As the larvae feed on maize roots, the plants lose their ability to absorb water and nutrients, significantly affecting their development (Gavloski et al. 1992). Damaged root systems can also lead to lodging, where the weakened maize plants become uprooted and fall over (Spike & Tollefson 1991). Such lodging often leads to significant yield losses if the fallen plants cannot be harvested. Severe corn rootworm damage to maize plants has been shown to reduce overall yield by as much as 40-50% (Urias-López & Meinke 2001). Costs due to yield losses, insecticide applications, and use of transgenic maize varieties have been estimated to total \$1 billion annually in the United States, making corn rootworm one of the most costly pests (Gray et al. 2009).

Recently, the genetic engineering of maize plants expressing Bt (*Bacillus thuringiensis* Berliner) proteins has allowed for effective suppression of corn rootworm in continuous maize production. Bt hybrids of maize have several important environmental benefits such as reduction in insecticide applications, high specificity for the target insects, and relatively few non-target effects (Rice 2004). In 2003, the first Bt maize expressing the Cry3Bb1 toxin was released for commercial use (EPA 2010a). The release of the Bt hybrids containing the Cry34/35Ab1 toxin and hybrids expressing the mCry3A toxin followed in 2007 (EPA 2010b,c). These toxins share similar modes of action against corn rootworm and bind to receptors in the midgut after tissue containing the toxins is ingested. This ingested toxin causes the formation of pores and eventual rupturing of the midgut, killing the insect (Schnepf 1998).

Use of Bt maize has led to the selection for resistance to Bt toxins in corn rootworm. This field-evolved resistance was first documented in *D. v. virgifera* populations in Iowa in 2009 in locations where fields were planted to Cry3Bb1 maize for at least three consecutive years (Gassmann et al. 2011). Since then, reports of unexpected root injury and high numbers of corn rootworm adults in Cry3Bb1 maize have been documented in Colorado, Illinois, Kansas, Missouri, Nebraska, South Dakota, and Wisconsin (DiFonzo et al. 2013, Wangila et al. 2015), with additional reports as far east as Michigan, New York, and Pennsylvania (Tooker 2014). These examples of damage to Bt maize have been associated with reports of severe lodging of the plants (Devos et al. 2013) and significant economic losses to producers (Gassmann 2012).

While damage to *Bt* maize was anecdotally reported for South Dakota, there were no published reports with quantified impact of corn rootworm to *Bt* maize in the state. Thus, we performed field experiments in eastern South Dakota to determine which *Bt* hybrids still suppressed corn rootworm damage to maize roots and decreased adult corn rootworm emergence. Hybrids expressing Cry3Bb1 have been the most commonly planted in South Dakota and were of particular interest, but we also tested the efficacy of mCry3A and Cry34/35Ab1 hybrids. Moreover, we surveyed the *Diabrotica* populations to determine the relative species composition of beetles in the state, given the anecdotal, but not previously published observation that *D. v. virgifera* seemed to have displaced *D. barberi* in areas with long-term use of *Bt* maize.

Materials and Methods

Field locations and history. Field experiments were conducted in Chester, South Dakota (43°52.836'N, 97°00.572'W) and Milbank, South Dakota (45°10.792'N, 96°33.924'W) in 2013 and 2014. Both locations were planted with maize hybrid YieldGard® expressing Cry3Bb1 toxin for more than five consecutive years. Each site had previous infestations of *D. v. virgifera* and *D. barberi*. The Chester location consisted of a 57 ha field, and the field areas not dedicated to our experiment were planted with Genuity® SmartStax® RIB Complete maize in 2013 and 2014. This refuge-in-a-bag (RIB) maize contained a blend of 95% Genuity® SmartStax® seed, which expresses both the Cry3Bb1 and Cry34/35Ab1 toxins targeting the rootworms, and 5% non-*Bt* seed. The field was cultivated through ridge-till, and fertilized with nitrogen, phosphorus, and potassium at a rate of 64-18-18 kg/ha. The Milbank location consisted of a 65 ha field, and field areas not dedicated to our experiment were planted with Optimum® AcreMax® Xtra maize in 2013 and 2014. This RIB maize contained a blend of 95% Optimum® AcreMax® Xtra seed, which expresses the Cry34/35Ab1 toxin targeting the rootworms, and 5% non-*Bt* seed.

Efficacy of *Bt* corn against corn rootworm. In both years the experiments included the following treatments: Cry3Bb1 maize (event MON88017, YieldGard®, hybrid DKC50-66, Monsanto, St. Louis, MO), mCry3A (event MIR604, Agrisure®, hybrid NK - N42Z, Syngenta Crop Protection, Greensboro, NC), Cry34/35Ab1 (event DAS-59122-7, hybrid P9917, Herculex®, Dow AgroSciences LLC, Indianapolis, IN and Pioneer Hi-Bred International, Incorporated, Johnston, IA), and a non-*Bt* refuge maize (hybrid DKC44-92) that served as a control. In Chester, experimental plots were planted on 14 May 2013 and 7 May 2014 using a 12-row planter with 76.2 cm row spacing and measured 4.6 m by 30 m in both years. The experiment was a completely randomized design with three replicates in 2013 and 2014. In Milbank, the experimental plots were planted on 16 May 2013 and 22 May 2014 using a 16-row planter with 76.2 cm row spacing and measured 6 m by 30 m in 2013 and 3 m by 30 m in 2014. The experiment was a completely randomized design with three replicates in 2013 and four replicates in 2014. At both locations, acetochlor (0.84 kg/L, Harness® herbicide, Monsanto, St. Louis, MO) was applied before maize seedling emergence, and glyphosate (0.48 kg/L, Roundup®, Monsanto, St. Louis, MO) was applied after seedling emergence in 2013 and 2014.

Plants within the non-Bt control plots were monitored for *D. virgifera* and *D. barberi* larval hatch by digging roots once a week starting at the V5 growth stage, continuing until third instars were found. When larvae reached third instar, four maize plants in two randomly selected locations within all of the experimental plots were cut off at 0.5 m above ground level. Emergence traps (60 by 60 by 60 cm, MegaView Science Co., Ltd., Taichung, Taiwan) were placed over the cut plants in order to capture emerging corn rootworm adults (i.e., two emergence traps were located in each of the plots). Adults in each individual emergence trap were collected every five days using a battery-operated aspirator (BioQuip, Rancho Dominguez, CA).

Feeding by corn rootworm larvae was evaluated on 8 and 9 August 2013, and 6 and 8 August 2014. In 2013, root systems were assessed from 10 plants randomly selected from the center rows of each plot. In 2014, the 10 plants selected for evaluation of root injury were first tested with lateral flow membrane strips to detect the presence of Cry3Bb1 and Cry34Ab1 toxins (QuickStix™ AS 015 LS and AS 054 LS, Envirologix™, Portland, ME). Each root mass was washed with a high pressure water hose (Greenworks 1700-PSI Electric Power Washer, Greenworks Tools, Mooresville, NC) and assessed for injury using a linear 0-3 node injury scale (Oleson et al. 2005), where a rating of 0 indicated no injury, and a rating of 3 indicated three or more nodes as completely destroyed by the larvae. Minor injury was assigned a rating of 0.05 to signify scarring of the roots caused by larval feeding (Oleson et al. 2005).

Root injury ratings and seasonal average adult counts from emergence traps were analyzed using ANOVA to test for differences among treatments in each of the two experiments (PROC GLM with Bt corn treatment as the factor in the model; SAS Institute 2011). Square root transformations were performed if needed to meet assumptions of normal distribution and homogeneity of variances. Data that did not meet assumptions of normal distribution and homogeneity of variances after transformations were analyzed using the Kruskal-Wallis test (Zarr 1999). Means separation tests were performed using parametric (Tukey's HSD) or non-parametric (Mann-Whitney) tests (Zarr 1999).

Species composition of *Diabrotica*. In order to survey populations of adults of the two species of *Diabrotica* emerging from the experiment plots and migrating from the surrounding fields at both locations, two sticky traps (14 by 23.5 cm, Great Lakes, IPM, Inc., Vestaburg, MI) were placed in each plot used for the Bt efficacy experiments described above. Sticky traps were affixed to bamboo sticks at 0.9 m above ground. Sticky traps were collected every five days, wrapped in clear plastic wrap, and corn rootworm adults were identified to species and counted in the laboratory. Abundance and diversity of *D. v. virgifera* and *D. barberi* adults were measured using these methods until the number of adults collected in each trap was zero for two collecting dates in succession, which typically occurred in early or mid-October of each year.

Abundance data from the sticky trap surveys were analyzed using the Friedman test (PROC FREQ statement with Bt corn treatments as a fixed factor and date as a block), as the data could not be transformed to satisfy the assumptions necessary for a repeated measures analysis of variance. All analyses were conducted in SAS 9.3 (SAS Institute 2011).

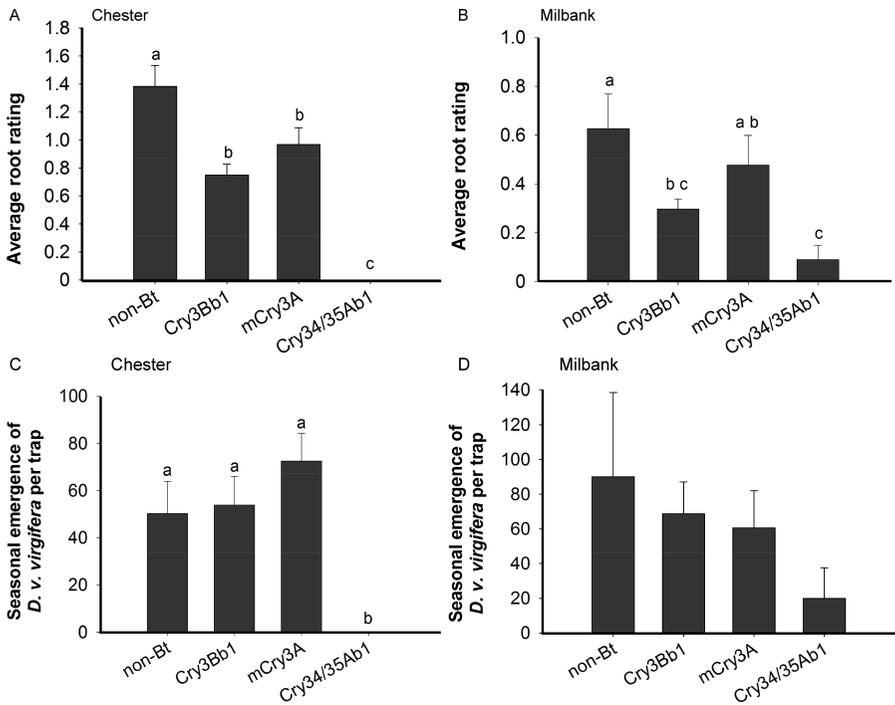


Fig. 1. Average root injury for maize treatments in Chester (A) and Milbank (B) and seasonal total emergence of *D. v. virgifera* per trap in Chester (C) and Milbank (D) in 2013. Bars represent treatment means \pm SEM. Means within each graph marked with different letters are significantly different (Tukey's HSD test, $P < 0.05$).

Results

Bt toxins expressed in the maize hybrids had significant effects on average injury of the roots caused by corn rootworm larvae. We found that at both locations in 2013 treatment had a significant effect on average root injury and emergence of adults (Figure 1). Overall, significantly higher root damage was observed in the non-Bt plots compared to the three Bt treatments in Chester ($X^2 = 9.8$; $df = 3$; $P = 0.02$; Figure 1A). Moreover, we noted less injury on the Cry34/35Ab1 maize in Chester compared to the two other maize hybrids expressing Bt toxins. There was virtually no damage to the roots of the Cry34/35Ab1 maize. In Milbank, on the other hand, maize expressing Cry3Bb1 and Cry34/35Ab1 sustained significantly less injury than non-Bt maize, while the average root damage to maize expressing mCry3A did not differ from non-Bt maize ($F = 5.35$; $df = 3, 8$; $P = 0.026$; Figure 1B). Average injury rating of the roots of the Cry3Bb1 and Cry34/35Ab1 maize were not statistically different from each other.

The impact of Bt treatments on the average emergence of *D. v. virgifera* per trap in 2013 differed between the two locations. In Chester, we noted significant effects of the treatments on the average number of beetles emerging from the

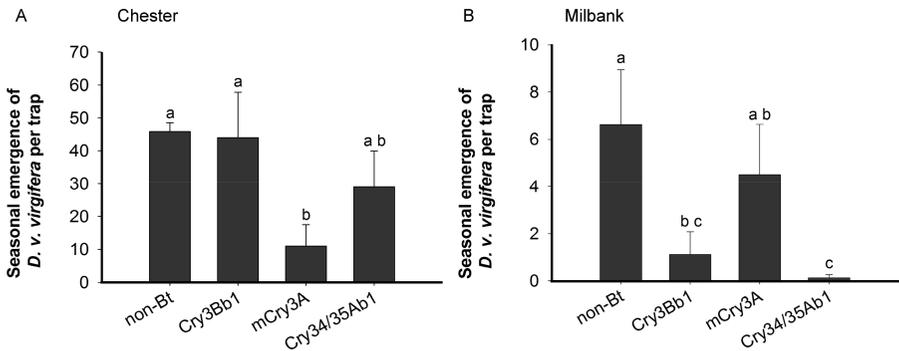


Fig. 2. Seasonal total emergence of *D. v. virgifera* per trap in Chester (A) and Milbank (B) in 2014. Bars represent treatment means \pm SEM. Means within each graph marked with different letters are significantly different (Tukey's HSD test, $P < 0.05$).

plots ($F = 8.19$; $df = 3, 8$; $P = 0.008$; Figure 1C). No beetles emerged from the plots planted to maize expressing the Cry34/35Ab1 toxin, while the number of *D. v. virgifera* recovered from the remaining plots was about equal for each treatment and averaged between 50-72 beetles per trap. However, treatment did not have any significant effects on the total emergence of *D. v. virgifera* from the plots at the Milbank location ($F = 1.8$; $df = 3, 8$; $P = 0.225$; Figure 1D). The numbers of beetles recovered from the Milbank plots averaged 20-90 beetles per trap.

Average root injury and seasonal total *D. v. virgifera* emergence were very low at both locations in 2014 (Figure 2). In fact, root injury was not discernable from zero at both locations and could not be analyzed statistically. We did note significant (or near significant) effects of Bt treatments on average emergence of *D. v. virgifera* at both locations in 2014 (Chester: $F = 3.40$; $df = 3, 8$; $P = 0.07$; Milbank: $F = 5.66$; $df = 3, 12$; $P = 0.012$). The highest numbers of beetles emerged from the non-Bt and Cry3Bb1 plots while the lowest number of beetles emerged from the mCry3A plots at Chester (Figure 2A). In Milbank, the greatest average emergence of adult *D. v. virgifera* was noted in the non-Bt and mCry3A plots, while the fewest beetles emerged from Cry34/35Ab1 plots (Figure 2B).

Surveys of populations of *D. v. virgifera* and *D. barberi* collected using sticky traps revealed significant differences in numbers of beetles of each species across both years and locations. There were strong general trends in relative abundance of the two species of *Diabrotica* that were consistent across the years and locations. In 2013, 99.9% of all beetles surveyed at the Chester location were *D. v. virgifera*, while the percentage of *D. barberi* was only 0.1%. Similar composition was detected at the Milbank location, where 100% of all beetles surveyed were *D. v. virgifera*. In 2014, the percentages of beetles surveyed at Chester were again 99.9% *D. v. virgifera* and 0.1% *D. barberi*. Relative abundance of *D. barberi* increased to 5.9% at Milbank in 2014, while of 94.1% of the beetles were identified as *D. v. virgifera*.

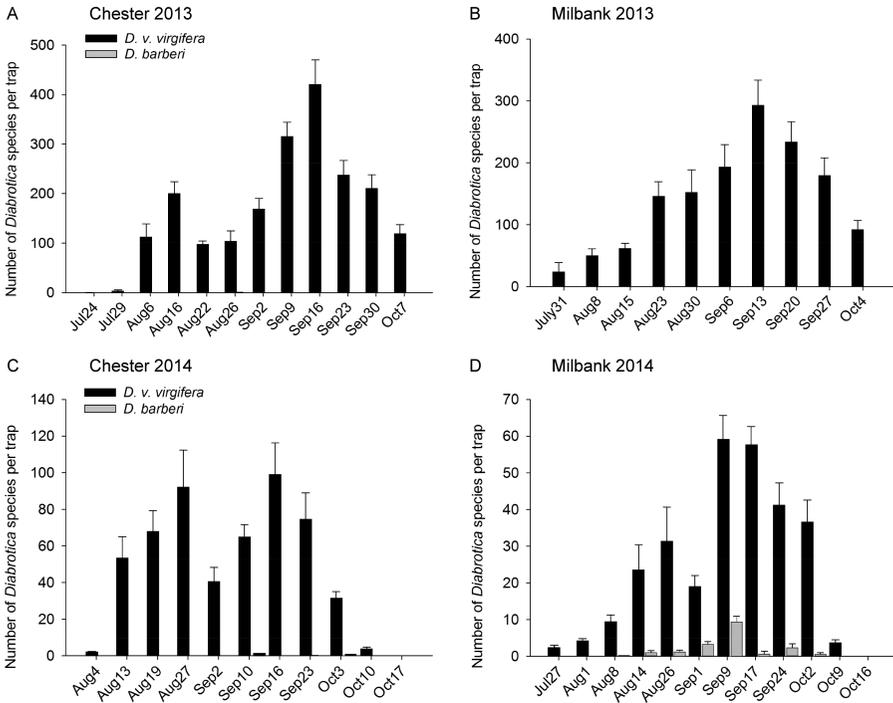


Fig. 3. Average number of *D. v. virgifera* and *D. barberii* captured over time in sticky traps at Chester (A) and Milbank (B) in 2013 and Chester (C) and Milbank (D) in 2014. Bars represent treatment means \pm SEM.

Captures of *D. barberii* occurred sporadically in both years and locations and the majority of beetles caught in sticky traps were *D. v. virgifera* (Figure 3). Numbers of *D. v. virgifera* were significantly greater than numbers of *D. barberii* in Chester in 2013 ($X^2 = 213.70$; $df = 1$; $P < 0.0001$). Averages of *D. v. virgifera* captured in Chester throughout the 2013 season followed a normal distribution, with the highest average of about 400 beetles captured on 16 September 2013 (Figure 3A). The capture of *D. barberii* only occurred on 26 August 2013 at this location (Figure 3A). Greater numbers of *D. v. virgifera* than *D. barberii* were also captured in Milbank ($X^2 = 197.37$; $df = 1$; $P < 0.0001$), with the highest average capture on 13 September 2013 (Figure 3B). No *D. barberii* were collected at this location in 2013.

While populations of the two *Diabrotica* species were lower in 2014 than in 2013, we again noted significantly greater numbers of *D. v. virgifera* than *D. barberii* in both locations (Chester: $X^2 = 189.17$; $df = 1$; $P < 0.0001$; Milbank: $X^2 = 247.17$; $df = 1$; $P < 0.0001$). The highest numbers of beetles were captured on 16 September 2014 at Chester (Figure 3C), and 9 September 2014 at Milbank (Figure 3D). We collected *D. barberii* at both locations in 2014, with the highest number of *D. barberii* captured in Chester on 10 September 2014 (Figure 3C). Captures of *D. barberii* occurred on eight sampling dates in Milbank, with the highest capture occurring on 9 September 2014 (Figure 3D).

Discussion

Injury to maize containing Cry3Bb1 and mCry3A toxins documented in this study implies that at least localized populations of corn rootworm in South Dakota can survive, complete development, and cause significant injury to Bt hybrids expressing Cry3Bb1 and mCry3A. Extremely cold winter temperatures and flooding during egg hatch likely contributed to an extensive reduction of corn rootworm population in 2014 and thus we have only one year of reliable data on corn rootworm damage to roots and emergence from Bt and non-Bt plots. *Diabrotica virgifera virgifera* was shown to be highly susceptible to the Cry3Bb1 toxins in laboratory (Siegfried et al. 2005) and field experiments (Al-Deeb & Wilde 2005) when Bt maize was first introduced for corn rootworm management on a large scale, but loss of efficacy has since been reported across the Corn Belt. This is the first published report of higher than expected injury to Cry3Bb1 corn in South Dakota. It is important to note that this experiment was not designed to explicitly test for resistance to Bt in corn rootworm populations or explore other mechanisms responsible for higher than expected injury to Bt corn. The outcomes of our study suggest, however, that the likelihood of resistance to Bt toxins in South Dakota is significant and should be examined.

If resistance of corn rootworms to Cry3Bb1 is indeed responsible for the injury we noted in our experiments, it might have also contributed to their survival on mCry3A maize. Cry3Bb1 and mCry3A are phylogenetically similar and belong to the same family of Cry toxin, and structural similarities between Cry3Bb1 and mCry3A may relate to the modes of action of these two toxins. Therefore, any mutations of Bt-binding sites in *D. v. virgifera* may result in simultaneous resistance of the beetles to both Cry3Bb1 and mCry3A (Gassmann et al. 2014). This phenomenon of cross-resistance may explain the unexpected injury to mCry3A in our experiments despite the fact that the beetles had not been exposed to this trait in locations where we tested it.

Cry34/35Ab1 maize provided greater protection than Cry3Bb1 and mCry3A against larval corn rootworm feeding and offered significantly better suppression of adult emergence in our experiments. Cry34/35Ab1 belongs to a family of Cry toxins that is structurally different from Cry3Bb1 and mCry3A Cry toxins (Gassmann et al. 2014). Previously published literature confirms high efficacy of Cry34/35Ab1 maize against corn rootworm larvae. For example, a seedling mat assay performed by Binning et al. (2010) indicated that 98.5% to 99.7% of susceptible corn rootworm were killed when fed Cry34/35Ab1 maize. Another example provided in Storer et al. (2006) illustrated that feeding on Cry34/35Ab1 maize caused 97.4% mortality in corn rootworm larvae.

It is likely that the outcomes of our experiment in 2014 were strongly affected by abiotic factors; specifically extremely cold temperatures and little snow cover during the winter and high soil moisture during corn rootworm egg hatch. Research has shown that eggs exposed to declining temperatures ranging from 0° to -7.5°C can result in higher mortality than eggs held at a constant temperature (Gustin 1986). Abundant soil moisture and in some cases severe flooding of the fields also likely contributed to high larval mortality. In a study by Spike & Tollefson (1988), adult emergence declined by 50% in locations of a field that had saturated topsoil for at least five days. This may explain significantly lower numbers of corn rootworm in 2014.

Our surveys of *Diabrotica* revealed that species composition has changed dramatically since the introduction of Bt maize. Studies performed before the widespread adoption of Bt maize in South Dakota indicate that *D. barberi* was much more abundant than *D. v. virgifera* in the state. For example, out of all *Diabrotica* species collected in Brookings County in 1995, 57% were *D. barberi* and 43% were *D. v. virgifera* (Ellsbury et al. 1998). The ratio of *D. barberi* to *D. v. virgifera* was even higher in 1996 with 86% *D. barberi* adults to 14% *D. v. virgifera* (Ellsbury et al. 1998). In contrast, we report that the relative abundance of *D. barberi* dropped to less than 1% and did not exceed 5.9%. The decline in *D. barberi* that we report may be related to the effect of extended diapause, a phenomenon where eggs of *D. barberi* diapause for more than one winter before hatching. Extended diapause has been documented in *D. barberi* populations in Minnesota, Iowa, and South Dakota (Levine et al. 1992), with some reports of diapause lasting three or more years (Mitchell & Onstad 2005). Extended diapause may slow the evolution of resistance, as prior generations of beetles experience less selection pressure, resulting in lower frequencies of resistance alleles in the larvae that hatch from the older eggs. This is one of several possible factors driving the apparent decline in populations of *D. barberi* and additional research is necessary before causal mechanisms are discerned.

Other studies have reported changes in herbivore diversity as a result of long-term Bt use, specifically due to decreased insecticide use. In some areas where Bt crops have replaced insecticides, secondary pests not targeted by Bt toxins have replaced primary pests that are successfully controlled by Bt toxins. For example, the western bean cutworm, *Striacosta albicosta* Smith (Lepidoptera: Noctuidae), is a secondary pest found throughout the United States Corn Belt and is generally not controlled by Bt maize (Dorhout & Rice 2010). *Striacosta albicosta* may have spread across the country as long-term Bt use erased intraguild competition with the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) and the corn earworm, *Helicoverpa zea* (Lepidoptera: Noctuidae), two pests that are highly susceptible to Bt toxins (Catarino et al. 2015).

Corn rootworm has proven to be highly adaptable to management practices and it is clear that long-term strategies to mitigate its impact on agriculture must be diverse. Bt traits need to be used in conjunction with multiple tactics to combat the pest and slow the rate of resistance to Bt technology. In most maize-growing areas, rotating to soybeans or another non-host crop can disrupt the life cycle of the pest and is a successful and sustainable method to control corn rootworm (Cullen et al. 2013). The use of soil insecticides may also prove effective (Gassmann 2012), as can rotation of Bt maize expressing different toxins to reduce selection pressure (Porter et al. 2012). Using pyramided maize hybrids that express more than one Bt toxin should also delay resistance to each of the toxins (Wangila et al. 2015). Regardless of what means of suppression of corn rootworm are used, a long-term sustainable strategy to manage this insect should be based on integrative approaches and frequent rotation of crops, Bt toxins, and judicious use of chemical control.

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